II. REMARKS

Formal Matters

Claims 1-5 and 10-20 are pending after entry of the amendments set forth herein.

Claims 1-5 and 14-20 are withdrawn from consideration.

Claims 6-13 were examined. Claims 6-13 were rejected.

Claims 10-12 are amended. The amendments to the claims were made solely in the interest of expediting prosecution, and are not to be construed as an acquiescence to any objection or rejection of any claim. Support for the amendments to the claims is found in the claims as originally filed, and throughout the specification, in particular at the following exemplary locations: page 23 line 25. Accordingly, no new matter is added by these amendments.

Claims 6-9 are canceled without prejudice to renewal, without intent to acquiesce to any rejection, and without intent to surrender any subject matter encompassed by the canceled claims. Applicants expressly reserve the right to pursue any canceled subject matter in one or more continuation and/or divisional applications.

Applicants respectfully request reconsideration of the application in view of the remarks made herein.

PTO 1449 form

The Applicants acknowledge receipt of the PTO 1449 form, initialed and dated by the Examiner, thereby indicating that the references cited in the Information Disclosure Statement filed in this application have been reviewed and made of record.

Rejections under 35 USC § 101 and 35 USC § 112, first paragraph - *Utility and Enablement Rejection Based on Assertion of Lack of Utility*

Claims 6-13 are rejected under 35 USC § 101, assertedly because the claimed invention is not supported by either a credible, specific and substantial asserted utility or a well established utility. The Office Action states that the application does not disclose the biological role of this VSHK-1 or its significance. Claims 35 USC § 112, first paragraph, are further rejected under 35 USC § 112, first paragraph assertedly because

one of skill in the art would not know how to use the claimed invention if its lacks utility. Since both rejections are established using the same reasons, Applicants respectfully traverse these rejections together.

Any rejection based on lack of utility should include a detailed explanation as to why the claimed invention has no specific and substantial credible utility[1] and whenever possible, the Office should provide documentary evidence.[1] In the absence of documentary evidence, the Office should provide a *prima facie* showing that establishes that it is more likely than not that a person skilled in the art would not consider credible any specific and substantial utility asserted by the Applicants for the claimed invention. A prima facie showing must contain the following elements: (1) an explanation that clearly sets forth the reasoning used in concluding that the asserted specific and substantial utility is not credible; (2) support for factual findings relied upon in reaching this conclusion; and (3) an evaluation of all relevant evidence of record.[1] A rejection based on lack of utility should not be maintained if an asserted utility for the claimed invention would be considered specific, substantial, and credible by a person of ordinary skill in the art in view of all evidence of record.[2]

In making this rejection, the Office Action states that it is clear from the instant specification the nucleic acid encoding the VSHK-1 polypeptide has been described as a chemokine receptor based on its similarity to known proteins. The Office Action appears to assert that because the specification provides no experimental evidence that VSHK-1 functions as a chemokine receptor, the utility is not specific and substantial.

On page 18 of the instant specification, it is stated that VSHK-1 exhibits one or more of the following biological activities: (1) Mediation of chemotaxis of immune system cells (e.g. neutrophils, lymphocytes, tumor-infiltrating, lymphocytes, hemopoietic progenitors, monocytes, natural killer cells); (2) involvement in angiogenesis or cell proliferation; and (3) involvement in glycosaminoglycan production.

Applicants respectfully assert that one of skill in the art would recognize that the claimed nucleic acids may be used to modulate chemotaxis of immune system cells. This assertion is supported by the post-filing publication of Schweickart et al., (*CCR11 is a*

[1] *Fed. Reg. Vol. 66 at page 1098, Section II-B, paragraph 3.*
[2] *Utility Examination Guidelines, Federal Register (Jan. 5, 2001) Vol. 66(4):1092-1099.*

functional receptor for the monocyte chemoattractant protein family of chemokines. J Biol Chem. 276:856, 2001; copy enclosed herewith), which describes the function of CCR11. CCR11 is identical to VSHK-1 (see the sequence alignment provided as Appendix A).

Scherickart et al. show that CCR11 is a functional receptor for a monocyte chemoattractant. Since CCR11 is a functional receptor for a monocyte chemoattractant, and CCR11 is identical to VSHK-1, VSHK-1 must also be a functional receptor for a monocyte chemoattractant. Accordingly, this biological activity of VSHK-1 described in the specification is confirmed by this publication.

Since the instant specification and Schweickart indicate the function of VSHK-1 as a monocyte chemokine receptor, and monocyte chemokine receptors have specific and substantial utility (e.g. for modulating monocyte chemotaxis), one of skill in the art would recognize that a nucleotide encoding VSHK-1 would have specific and substantial utility.

Applicants submit that the rejection of claims 6-13 under 35 USC § 101 and 35 USC § 112 has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

Rejections under 35 USC § 112, first paragraph - *Enablement*

Claims 6-13 are rejected under 35 U.S.C. § 112, first paragraph, assertedly because the specification does not reasonably provide enablement for the claimed nucleic acids. Applicants respectfully traverse the rejection as it may be applied to the present claims.

Without conceding that the grounds for the rejections in the Office Action are correct, the claims, as amended, are directed to an isolated polynucleotide containing at least about 1500 contiguous nucleotides of SEQ ID NO:1.

The law regarding enablement of inventions is clear: "[t]he test of enablement is whether one reasonably skilled in the art could make or use the invention from the

disclosures in the patent coupled with information known in the art without undue experimentation."[3]

To aid in determinations of enablement, courts have identified eight factors for consideration: (a) the quantity of experimentation necessary; (b) the amount of direction or guidance presented; (c) the presence or absence of working examples; (d) the nature of the invention; (e) the state of the prior art; (f) the relative skill of those in the art; (g) the predictability or unpredictability of the art; and (h) the breadth of the claims.[4]

The claimed isolated polynucleotides contain at least about 1500 contiguous nucleotides of SEQ ID NO:1, which is shown in Figure 1 of the instant application. One of skill in the art would recognize that several working examples of polynucleotides containing 1500 contiguous nucleotides of SEQ ID NO:1 are shown in this figure. For example, one fragment of 1500 contiguous nucleotides is represented by nucleotide positions 1-1500 of SEQ ID NO:1, and a different fragment of 1500 contiguous nucleotides of SEQ ID NO:1 is represented by nucleotide positions 2-1501 of SEQ ID NO:1. One of skill in the art would recognize that by changing the start position of the fragment in SEQ ID NO:1, a large number of different polynucleotide fragments containing at least about 1500 contiguous nucleotides of SEQ ID NO:1 may be obtained. In other words, since SEQ ID NO:1 is 1958 contiguous nucleotides in length, there are at least approximately 458 (i.e. 1958-1500) working examples of polynucleotides containing at least about 1500 contiguous nucleotides of SEQ ID NO:1.

Furthermore, one of skill in the art would recognize that a large number of species of the claimed polynucleotide encompass the entire coding sequence of SEQ ID NO:1 (encoded by nucleotides 84-1131 of SEQ ID NO:1; a total of 1047 contiguous nucleotides in length), and, as such, would also recognize that no undue experimentation would be required to, for example, use those polynucleotides to express a full length VSHK protein.

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3 *United States v. Telectronics, Inc.*, 8 USPQ 2d 1217, 1233 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989). See also *Genentech, Inc. v. Novo Nordisk*, 42 USPQ 2d 1001 (Fed. Cir. 1997), *cert. denied*, 522 U.S. 963 (1997); *Scripps Clinic and Research Foundation v. Genentech, Inc.*, 18 USPQ 2d 1001 (Fed. Cir. 1991).

4 *Ex Parte Forman.*, 230 USPQ 546, 547 (Bd.Pat.App & Interf. 1986); and, *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

Since a large number of working examples of polynucleotides containing at least about 1500 contiguous nucleotides of SEQ ID NO:1 are provided in the specification, and one of skill in the art would be able to use a significant number of such polynucleotides to express functional polypeptide, one of skill in the art would practice the invention without undue experimentation.

Applicants respectfully submit that the specification provides an enabling disclosure and an adequate description of an isolated polynucleotide containing at least about 1500 contiguous nucleotides SEQ ID NO:1. The Examiner is thus respectfully requested to withdraw the rejection.

Rejections under 35 USC § 112, first paragraph – *Written Description*

Claims 6-13 are further rejected under 35 U.S.C. § 112, first paragraph, as assertedly containing subject matter which was not described in the specification in such a way as to reasonable convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Applicants respectfully traverse the rejection as it may be applied to the present claims.

Without conceding that the grounds for the rejections in the Office Action are correct, the claims, as amended, are directed to an isolated polynucleotide containing at least about 1500 contiguous nucleotides of SEQ ID NO:1.

The standard for written description has been established over several years of court cases such as *Vas-Cath Inc. v. Mahurkar*[5] and *In re Wertheim*[6] and has culminated in the publication of the "Written Description Guidelines" Federal Register Vol. 66 No. 4, dated January 5, 2001 to which the Office must adhere to when making a written description determination. The law of written description does not require that the specification specifically describe all species that are encompassed by the claims.

A landmark and often cited case involving written description of nucleic acid invention is *Regents of the University of California v. Eli Lilly & Co*[7], hereafter "Lilly". Lilly states that:

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5 *Vas-Cath Inc. v. Mahurkar*, 19 USPQ2d 1111 (Fed. Cir. 1991).
6 *In re Wertheim* 191 U.S.P.Q. 90 (C.C.P.A. 1996)
7 *Regents of the University of California v. Eli Lilly & Co* 119 F.3d 1559 (Fed. Cir. 1997) at 1568-69

> "A description of a genus of cDNAs may be achieved by means of a recitation of **a representative number of cDNAs**, defined by nucleotide sequence, falling within the scope of the genus **or** of a recitation of **structural features** common to the members of the genus." (emphasis added)

As such, according to the Law, the written description requirement for a genus of nucleic acids may be satisfied by a) a representative number of species, **or** b) a recitation of structural features common to all members of the genus.

Applicants respectfully submit that the specification, as filed, describes the claimed genus of nucleic acids by both describing a representative number of species **and** reciting structural features common to all members of the genus.

As discussed above, the claimed isolated polynucleotides contain at least about 1500 contiguous nucleotides of SEQ ID NO:1, which is shown in Figure 1 of the instant application. One of skill in the art would recognize that several examples of polynucleotides containing 1500 contiguous nucleotides of SEQ ID NO:1 are described in this figure. For example, one fragment of 1500 contiguous nucleotides is described by nucleotide positions 1-1500 of SEQ ID NO:1, and a different fragment of 1500 contiguous nucleotides of SEQ ID NO:1 is described by nucleotide positions 2-1501 of SEQ ID NO:1. One of skill in the art would recognize that by changing the start position of the fragment in SEQ ID NO:1, a large number of different polynucleotide fragments containing at least about 1500 contiguous nucleotides of SEQ ID NO:1 are described. In other words, since SEQ ID NO:1 is 1958 contiguous nucleotides in length, Fig. 1 describes at least 458 (i.e. 1958-1500) examples of polynucleotides containing at least about 1500 contiguous nucleotides of SEQ ID NO:1. Applicants respectfully submit that the description of at least 458 polynucleotide species encompassed by the claim is a sufficient number to meet the requirements for written description.

Applicants further submit that all species of the claimed genus have a structural feature in common: they all have at least 1500 contiguous nucleotides of SEQ ID NO:1.

As such, Applicants respectfully submit that the specification, as filed, describes a representative number of the claimed species *and* recites structural features common to all members of the genus and, thus, the specification meets the requirements of 35 U.S.C. § 112, first paragraph (written description).

Applicants respectfully submit that the specification provides an adequate description of an isolated polynucleotide containing at least about 1500 contiguous nucleotides SEQ ID NO:1. The Examiner is thus respectfully requested to withdraw the rejection.

Rejection under 35 USC § 112, second paragraph

The Office Action stated that that claim 9 is rejected under 35 USC § 112 as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 9 has been cancelled without prejudice, and, as such, Applicants respectfully submit that this rejection is moot.

Rejection under 35 USC § 102(e)

The Office Action stated that claims 6-13 are rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent No. 5,932,455 (Lal). Applicants respectfully traverse the rejection as it may be applied to the present claims.

The claims, as amended, are directed to an isolated polynucleotide containing at least about 1500 contiguous nucleotides of SEQ ID NO:1.

Lal fails to disclose an isolated polynucleotide containing at least about 1500 contiguous nucleotides of SEQ ID NO:1, and, as such, fails to anticipate the claimed invention.

Applicants submit that the rejection of claims 6-13 under 35 U.S.C. 102 has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

Rejection under 35 USC § 102(b)

The Office Action stated that claims 6-13 are rejected under 35 U.S.C. § 102(b) as being anticipated by Matsuoka et al. (1993). Applicants respectfully traverse the rejection as it may be applied to the present claims.

The claims, as amended, are directed to an isolated polynucleotide containing at least about 1500 contiguous nucleotides of SEQ ID NO:1.

Matsuoka fails to disclose an isolated polynucleotide containing at least about 1500 contiguous nucleotides of SEQ ID NO:1, and, as such, fails to anticipate the claimed invention.

Applicants submit that the rejection of claims 6-13 under 35 U.S.C. 102 has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

III. CONCLUSION

Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number 2300-1544.

Respectfully submitted,
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Date: Feb 25, 2003

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CCR11 Is a Functional Receptor for the Monocyte Chemoattractant Protein Family of Chemokines*

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Chemokines mediate their diverse activities through G protein-coupled receptors. The human homolog of the bovine orphan receptor PPR1 shares significant similarity to chemokine receptors. Transfection of this receptor into murine L1.2 cells resulted in responsiveness to monocyte chemoattractant protein (MCP)-4, MCP-2, and MCP-1 in chemotaxis assays. Binding studies with radiolabeled MCP-4 demonstrated a single high affinity binding site with an IC_{50} of 0.14 nM. As shown by competition binding, other members of the MCP family also recognized this receptor. MCP-2 was the next most potent ligand, with an IC_{50} of 0.45 nM. Surprisingly, eotaxin (IC_{50} = 6.7 nM) and MCP-3 (IC_{50} = 4.1 nM) bind with greater affinity than MCP-1 (IC_{50} = 10.7 nM) but only act as agonists in chemotaxis assays at 100-fold higher concentrations. Because of high affinity binding and functional chemotactic responses, we have termed this receptor CCR11. The gene for CCR11 was localized to human chromosome 3q22, which is distinct from most CC chemokine receptor genes at 3p21. Northern blot hybridization was used to identify CCR11 expression in heart, small intestine, and lung. Thus CCR11 shares functional similarity to CCR2 because it recognizes members of the MCP family, but CCR11 has a distinct expression pattern.

Chemokines are a family of small proteins, usually 70–90 amino acids in length, that are responsible for the directed migration of specific cell types (for reviews, see Refs. 1–6). The complexity and functions of the chemokine family, now with more than 30 genes, have become increasingly diverse as more members have been identified and characterized. Chemokines play a critical role in the host response to infection because they are responsible for recruitment of leukocyte subsets to sites of pathogen entry (7, 8). Many inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and asthma (9), have been associated with elevated chemokine expression. In addition, chemokines are also responsible for the migration of cells within certain lymphoid organs that are critical for leukocyte development, such as thymus (10–12), lymph node (13), and spleen (14, 15). As shown by gene targeting studies, the chemokine stromal cell-derived factor (SDF)-1 is critical for proper neuronal and cardiac development (16, 17). Chemokines have also been implicated in cardiovascular processes such as angiogenesis and atherosclerosis (18).

Chemokines are recognized by specific seven transmembrane-spanning, G protein-coupled receptors (GPCRs)[1] (for review, see Refs. 19 and 20). Previously characterized chemokine receptors share significant homology, with 25–65% identical amino acids, and consequently form their own branch of the GPCR family tree. Many chemokine receptors were originally identified as orphan GPCRs. There remain several orphan GPCRs with high similarity to the chemokine receptor family.

The orphan receptor PPR1 was originally isolated from bovine papillary tissue in a search for gustatory receptors (21). However, the expression of PPR1 appears to be higher in lung than in tongue. In addition, PPR1 shares more similarity to chemokine receptors than gustatory or olfactory receptors. Because of this similarity, we isolated a human homolog of PPR1 and examined its ability to function as a chemokine receptor. The human homolog binds members of the MCP family (MCP-1, MCP-2, MCP-3, MCP-4, and eotaxin) with high affinity and also mediates responses to MCP-4, MCP-2, and MCP-1 in chemotaxis assays. In accordance with the Chemokine Nomenclature Committee, we have designated this receptor CCR11.

EXPERIMENTAL PROCEDURES

Materials and Reagents—The chemokines IL-8, IP-10, I-309, SDF-1, MIP-1α, MIP-1β, MCP-3, MCP-4, ELC (also known as MIP-3β), SLC (6Ckine), NAP-2, ENA-78, HCC-4, HCC-1, LKN-1 (MIP-5 or HCC-2), lymphotactin, and fractalkine were purchased from R & D Systems (Minneapolis). MCP-1, PARC, MDC, TARC, and eotaxin were purchased from Gryphon Sciences (So. San Francisco, CA). PF-4, MCP-2, MGSA, MIG, RANTES, TECK, and LARC (MIP-3β) were purchased from Peprotech (Rocky Hill, NJ).

Isolation of CCR11 cDNA and Gene—The GenBank Expressed Sequence Tag (EST) data base was searched with the bovine PPR1 cDNA sequence (21) using the BLAST algorithm (22). Three human ESTs were identified (H67224, AA215577, AI131555) with high homology to the bovine sequence. The clone H67224 was obtained from Research Genetics (Huntsville, AL), and the entire insert was sequenced. Because this EST contained only a fragment of the coding region, additional cDNA libraries were screened. Three human cDNA libraries were hybridized with a probe from the EST sequence (prepared by polymerase chain reaction amplification with the primers 5′-GTCTCTGGAATGCAGTTTCTGG and 5′-CGATGTCCATGCGTTTGCTCA): small intestine (Stratagene, La Jolla, CA), macrophage (described by 23), and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF193507.

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[1] The abbreviations used are: GPCR(s), G protein-coupled receptor(s); bp, base pairs; CCR, CC chemokine receptor; ELC, EBI1-ligand chemokine; ENA-78, epithelial cell-derived neutrophil-activating protein; EST, expressed sequence tag; LARC, liver and activation-regulated chemokine; LKN-1, leukotactin-1; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; NAP-2, neutrophil-activating protein-2; PARC, pulmonary and activation-regulated chemokine; MIG, monokine induced by interferon γ; IL-8, interleukin-8, IP-10, interferon γ-inducible protein-10, MGSA, melanocyte growth-stimulating activity; PF-4, platelet factor-4; RANTES, regulated on activation, normal T cell expressed and secreted; SDF, stromal cell-derived factor; SLC, secondary lymphoid tissue chemokine; TARC, thymus and activation-regulated chemokine; TECK, thymus-expressed chemokine; HCC, hemofiltrate CC chemokine.

peripheral blood mononuclear cell (phorbol myristate acetate/ionomycin-stimulated, 24). More than a million clones were examined in each library. No clones were found in the macrophage library. A single clone was identified in the peripheral blood mononuclear cell library which was 1388 bp in length and lacked 188 bp of the amino-terminal coding sequence. Five clones were isolated from the small intestine cDNA library, ranging in size from 131 to 1153 bp. The consensus cDNA sequence was missing 14 bp from the 5'-end of the coding region when aligned with the bovine PPR1 coding region sequence. To determine the amino-terminal coding region, a human genomic P1 library (Genome Systems Inc., St. Louis) was screened by polymerase chain reaction with the above primers to isolate the CCR11 gene. The 5'-coding region of the isolated clone was sequenced with primers based on the cDNA sequence. The deduced genomic sequence provided the remaining coding sequence for CCR11. The genomic sequence presented in Fig. 1 (residues 1–275) appears to contain no intervening sequences because it has contiguous homology with the bovine cDNA sequence (21). Four nucleotide differences were identified, one of which resulted in an amino acid change at position 143 (lysine, in the genomic and small intestine clones, to asparagine, in the peripheral blood mononuclear cell clone).

CCR11 Expression—The CCR11 coding region was amplified from the P1 clone with primers 5'-GCCCAAGCTTGCCACCATGGCTTTG-GAACAGAACCAGTCAAC and 5'-CTAGTCTAGAGTATCCAAGCAAA-AGGCAGAGCAG, which included *Hin*dIII and *Xba*I cloning sites. This fragment was inserted into pNEF6, a vector containing the Chinese hamster elongation factor-1α gene promoter and neomycin resistance gene. The amplified coding region of CCR11 was sequenced to ensure that no mutations were introduced by polymerase chain reaction. The expression construct was transfected into mouse pre-B L1.2 cells by electroporation with 10 μg of plasmid at 250 V, 960 microfarads, 72 ohm resistance using a Gene Pulser (Bio-Rad). Transfectants were selected and expanded in 800 μg/ml G418. These cells were used in a chemotaxis assay against a panel of chemokines (1 nM and 10 nM each) in order to identify ligands for CCR11 and to isolate CCR11-expressing transfectants (see under "Chemotaxis Assays"). Transfected cells that migrated were collected, cloned by limiting dilution, and expanded for further analysis.

Chromosomal Localization—A genomic P1 clone of approximately 90-kilobase pairs containing the human CCR11 gene was labeled with digoxigenin dUTP by nick translation and used as a probe for fluorescence *in situ* hybridization of human chromosomes (Genome Systems, Inc.). The labeled probe was hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes. Reactions were carried out in the presence of sheared human DNA in 50% formamide, 10% dextran sulfate, 30 mM sodium chloride, 3 mM sodium citrate, and 0.1% SDS. Hybridization signals were detected by treating slides with fluoresceinated anti-digoxigenin antibodies followed by counterstaining with 4,6-diamidino-2-phenylindole. Initial hybridization resulted in specific labeling of the middle long arm of a group A chromosome believed to be chromosome 3 on the basis of size, morphology, and banding pattern. A labeled genomic probe that is specific for the centromere of chromosome 3 was co-hybridized with the CCR11 probe and detected with Texas Red avidin. 80 metaphase cells were analyzed, with 72 exhibiting specific labeling.

Northern Blot Analysis—The expression of CCR11 mRNA was examined by Northern blot analysis. A human multi-tissue Northern blot was purchased from CLONTECH and hybridized as described (25). A gel-purified fragment containing most of the coding region of human CCR11 (1388 bp) was used as a hybridization probe.

Chemotaxis Assays—Cell migration was assayed using L1.2 cells stably transfected with CCR11 cDNA. Approximately 10^6 cells resuspended in 0.1 ml of RPMI 1640 medium with 0.5% bovine serum albumin (endotoxin-reduced, Intergen, Purchase, NY) were loaded in the upper wells of a transwell chamber (3-μm pore size, 6.5-mm diameter, Costar, Cambridge, MA). Test chemokines at the concentrations indicated were added to the lower wells in a volume of 0.6 ml. After 4 h at 37 °C, cells that migrated to the lower chamber were collected and counted using a fluorescence-activated cell sorter (Becton-Dickinson, Franklin Lakes, NJ). Values are expressed as the chemotaxis index, which is the ratio of cells that migrated toward chemokine divided by cells that migrated toward buffer alone.

Calcium Mobilization—Cells were suspended at 3×10^6 cells/ml in complete RPMI medium with 10% fetal bovine serum. Cells were incubated with 1 μM fura-2/AM (Molecular Probes, Eugene OR) at room temperature for 30 min in the dark. After washing, cells were resuspended at 2×10^6 cells/ml in phosphate-buffered saline. To measure intracellular calcium, cells in 2 ml were placed in a quartz cuvette in an SLM Aminco-Bowman series 2 luminescence spectrometer. Fluorescence was monitored at 340 nm (excitation wavelength 1), 380 nm (excitation wavelength 2), and 510 nm (emission wavelength). Chemokines were added at 100 nM final concentration.

Binding Assays—For binding experiments, 5×10^5 CCR11 transfected L1.2 cells were incubated for 1 h at room temperature with 0.1 nM ^{125}I-MCP-4 (NEN Life Science Products) in the presence or absence of various concentrations of chemokines in 200 μl of binding buffer (25 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% bovine serum albumin). Following incubation, cells were transferred to poly(ethyleneimine)-coated GF-B 96-well plates and washed three times with wash buffer (25 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5 M NaCl). Scintillant was added to each well, and bound ligand was quantified using a Wallac 1450 Microbeta Liquid Scintillation Counter (Gaithersburg, MD). Binding competition curves were fitted using a four-parameter logistic equation (GraphPad Prism, GraphPad Software, San Diego). Values were converted to percent ^{125}I-MCP-4 bound with 100% being the number of counts with no competing chemokine (2400 cpm) and 0% being background binding (in the presence of 1 μM unlabeled MCP-4, 440 cpm).

RESULTS

Isolation of the Human Gene for PPR1—Matsuoka and colleagues (21) previously isolated an orphan GPCR from bovine taste papillary tissue. Hydropathy and sequence analyses demonstrated that PPR1 was a member of the GPCR superfamily. More recent homology comparisons suggested a closer relationship to chemokine receptors than gustatory or olfactory receptors. Three human EST cDNA sequences were identified in the GenBank data base with high homology to the bovine PPR1 sequence. Oligonucleotide primers were designed from the human sequences and used to identify six partial cDNA clones and a genomic P1 clone of approximately 90 kilobase pairs which contained the entire gene sequence. Based on the functional data below, we have designated this human gene CCR11.

The CCR11 DNA sequence and encoded amino acid sequence are presented in Fig. 1. Hydropathy analysis (not shown) delineated seven hydrophobic domains typical of a seven-transmembrane spanning GPCR. Human CCR11 is 86% identical to bovine PPR1 at the amino acid level. This high degree of similarity is consistent with other GPCR genes when compared across mammalian species. Like most GPCRs, CCR11 contains potential *N*-linked glycosylation sites, two in the amino-terminal extracellular domain and one in the third extracellular loop. Similar to other chemokine receptor sequences, CCR11 contains single cysteine residues in each of the four predicted extracellular domains. As shown in Fig. 2, CCR11 shares 28–36% identity with other human chemokine receptors. The receptor with highest homology to CCR11 is CCR7 (36% identical at the amino acid level) followed by CCR6 and CCR9 (each 33% identical to CCR11). CCR11 is less homologous to other members of the GPCR superfamily, the next closest being lipid mediator receptors (platelet- activating factor receptor, 24%; leukotriene B4 receptor, 22%) and the chemotactic peptide receptor (fMet-Leu-Phe receptor, 19%). Like many other GPCR genes, the coding region of the CCR11 gene contains no intervening sequences.

Chromosomal Localization of CCR11—Many GPCR genes are clustered in the human genome. Indeed, the genes for the majority of the CC chemokine receptors are encoded at 3p21 (25, 26). Because clusters of genes are generally functionally related, we identified the chromosome location of the CCR11 gene. The human P1 clone containing the CCR11 gene was used as a probe for fluorescence *in situ* hybridization to human chromosomes. The results are presented in Fig. 3, where the CCR11 probe signal is green, and a specific chromosome 3-centromere probe signal is red. Measurement of specifically labeled chromosomes demonstrated that the CCR11 gene is located at a position that is 42% the distance from the

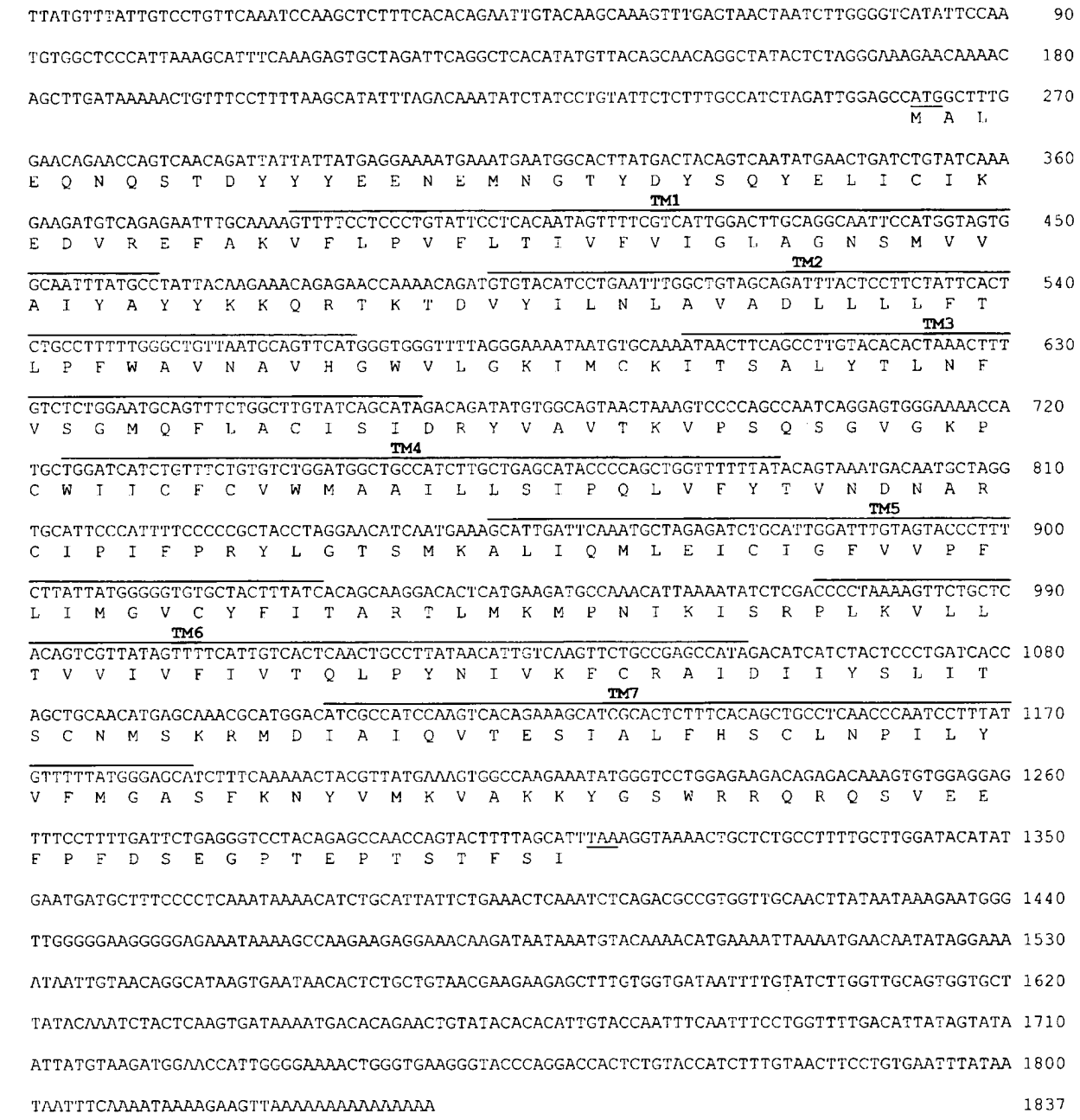

FIG. 1. **CCR11 DNA sequence.** The CCR11 DNA sequence was compiled from six cDNA clones and a genomic P1 clone. The deduced amino acid sequence is shown below the DNA sequence. Putative transmembrane domains are indicated.

centromere to the telomere of chromosome arm 3q, an area that corresponds to 3q22.

Northern Blot Analysis—To determine sites of expression of CCR11, Northern blot hybridizations were performed. The CCR11 gene was used as a hybridization probe for 12 different human tissues. CCR11 was expressed most abundantly in human heart, small intestine, and lung (Fig. 4). Lower levels of hybridization were observed in kidney, liver, and colon. The size of the primary transcript is approximately 2000 bases, which corresponds well with the cDNA size. The most abundant transcript in heart appears to be of greater size than that seen in other tissues and perhaps represents an alternatively spliced transcript.

Functional Responses of CCR11 Transfectants—Murine L1.2 cells were transfected with CCR11 and then tested for chemotaxis to a panel of 29 human chemokines. This panel included MIP-1α, MIP-1β, RANTES, MCP-1, MCP-2, MCP-3, MCP-4, eotaxin, ELC, SLC, LARC, PARC, MDC, TARC, TECK, IL-8, IP-10, I-309, SDF-1, MGSA, MIG, NAP-2, ENA-78, PF-4, HCC-1, HCC-4, LKN-1, lymphotactin, and fractalkine. Each chemokine was tested at 1 nM and 10 nM because these concentrations are generally optimal for other chemokine-chemokine receptor combinations. The most significant migration was observed to MCP-4, with some chemotaxis also observed toward MCP-2 and MCP-1. No other chemokines induced significant cell migration. The CCR11 transfectants that migrated toward MCP-4 were harvested, cloned by limiting dilution, and expanded for further functional studies. As shown in Fig. 5, CCR11 transfectants selected in this manner were tested in chemotaxis assays with a range of concentrations of MCP-4,

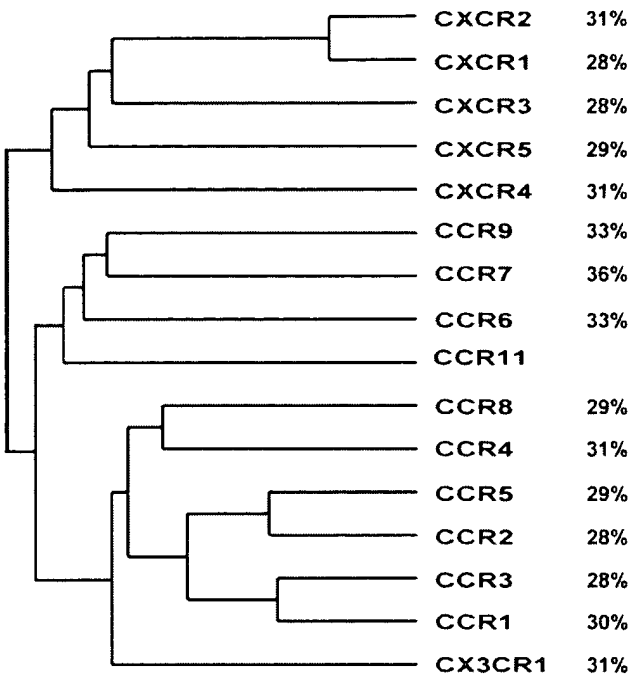

FIG. 2. **Comparison of CCR protein sequences.** This dendrogram analysis illustrates the similarity of the deduced amino acid sequence of CCR11 with other CCRs. Percentages of identity with CCR11 are shown to the *right*.

MCP-1, MCP-2, MCP-3, and eotaxin. Confirming the original observation, CCR11 transfectants migrated most efficiently toward MCP-4, with peak chemotaxis occurring at 10 nM. Significant migration was also observed toward MCP-2 and MCP-1 with peak chemotaxis occurring at 10–100 nM, although the number of cells migrating was slightly less than MCP-4. MCP-3 and eotaxin functioned as agonists only at the highest concentration of 1 μM.

CCR11 transfectants were also tested for calcium mobilization in response to ligand stimulation. Small but significant calcium flux was observed when transfectants were stimulated with MCP-4 (results not shown). This response was quantitatively not as strong as we have observed previously with other chemokine receptor-ligand pairs (see "Discussion"). No significant calcium flux was observed in response to MCP-1 or MCP-2 stimulation.

Receptor Binding Assays—Because MCP-4 was the most potent functional ligand, radiolabeled MCP-4 was used as a probe to examine binding to CCR11 transfected L1.2 cells. As shown in Fig. 6*A*, the ^{125}I-MCP-4 binding was inhibited competitively with increasing concentrations of unlabeled MCP-4 (IC_{50} of 0.140 nM), MCP-2 (IC_{50} of 0.458 nM), MCP-3 (IC_{50} of 4.08 nM), eotaxin (IC_{50} of 6.72 nM), or MCP-1 (IC_{50} of 10.7 nM). This suggests that all five ligands recognize a common binding site on CCR11 and that MCP-4 exhibits the greatest affinity. The observed binding of MCP-4, MCP-2, and MCP-1 is consistent with the functional chemotactic responses described above. However, MCP-3 and eotaxin bind with reasonable affinity but only act as agonists at more than 100-fold higher concentrations.

To examine specificity of binding to CCR11, 17 additional chemokines were tested at 1000-fold molar excess for competition of radiolabeled MCP-4 binding. The MCP family members, including eotaxin, effectively competed with ^{125}I-MCP-4 for binding to CCR11 (Fig. 6*B*). The other chemokines did not compete for CCR11 binding even at this high concentration.

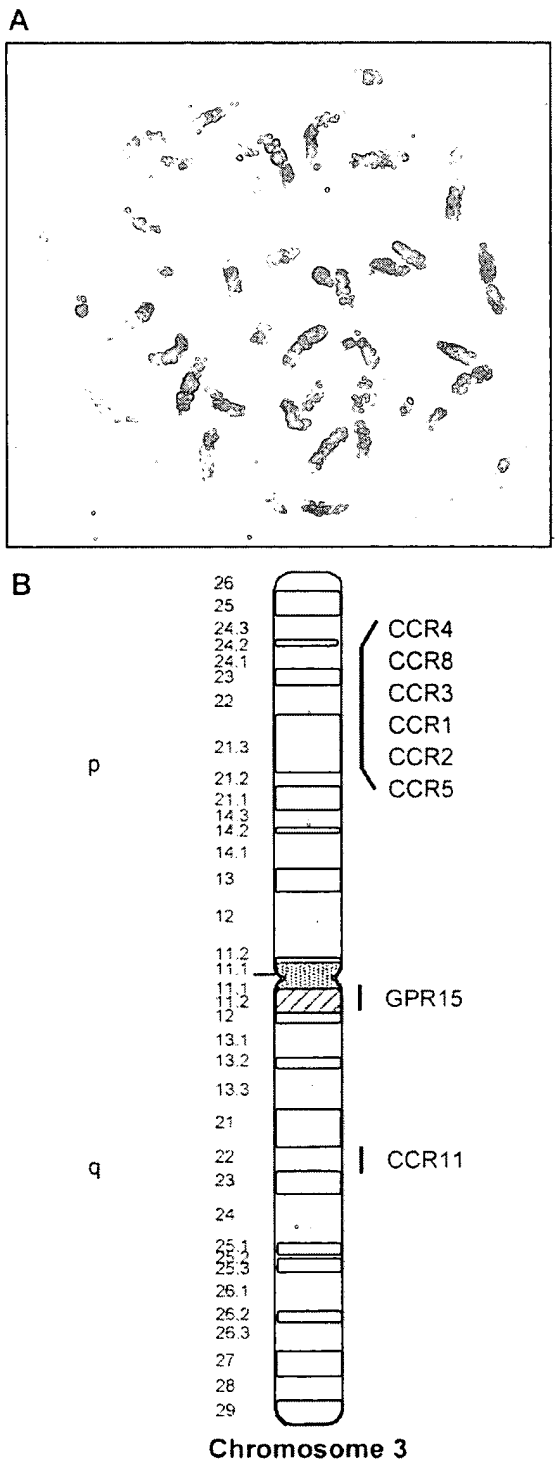

FIG. 3. **Localization of human CCR11 to chromosome 3.** *Panel A*, fluorescent *in situ* hybridization of human metaphase chromosomes to the CCR11 genomic DNA probe (*green dots*). Chromosomal identification was confirmed with a specific probe for the chromosome 3 centromere (*red*). *Panel B*, idiogram illustrating the chromosomal position of the CCR11 gene at 3q22.

DISCUSSION

CCR11 was identified during a search of the human EST data base for homologs of the bovine orphan PPR1. When the full coding region of CCR11 was assembled, it was found to be 86% identical to PPR1 at the amino acid level. Homology com-

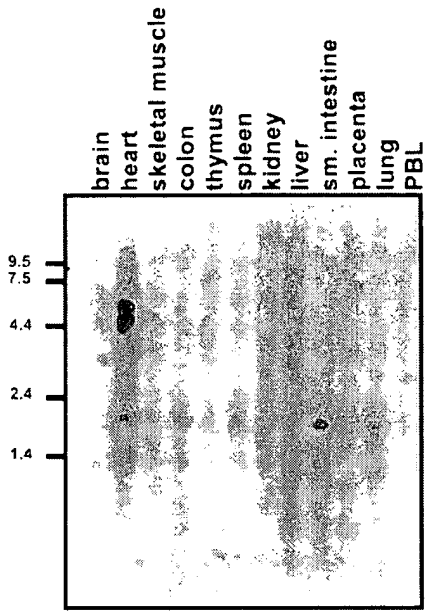

FIG. 4. **Tissue distribution of CCR11 expression.** Northern blot analysis of human tissue RNA hybridized with the CCR11 probe. Standard sizes in kilobases are indicated to the *left*.

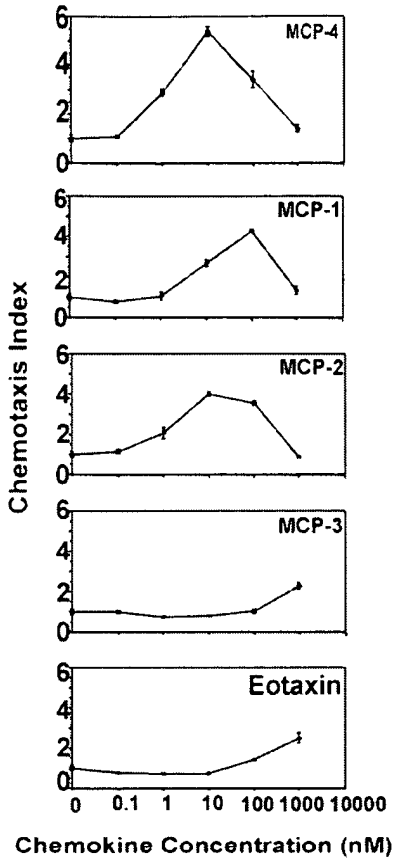

FIG. 5. **Chemotaxis of CCR11 transfectants.** A transwell assay was used to measure chemotaxis of L1.2 cells transfected with CCR11. Cells were placed in the upper wells. MCP-1, MCP-2, MCP-3, MCP-4, or eotaxin was in the lower wells at the indicated concentrations. Migrated cells were collected and counted by fluorescence-activated cell sorting. These results are expressed as mean ± S.E. and are representative of three separate experiments.

parisons indicated that CCR11 is most closely related to chemokine receptors. Its closest relatives are CCR7 (36% identical), CCR6 (33%), and CCR9 (33%). Chromosomal mapping of CCR11 localized it to 3q22. Interestingly, many other CC chemokine receptors also map to chromosome 3, including CCR1, CCR2, CCR3, CCR4, CCR5, and CCR8 (26). CCR11, however, is significantly separated from these receptors, which are clustered at 3p21–24. This suggests that CCR11 is more distantly related to most CC chemokine receptors, consistent with the sequence homology comparisons presented in Fig. 2. The CCR11 gene maps somewhat closer to the orphan receptor GPR15 (27; also known as BOB, 28) which is located at 3q11.2–13.1 (27).

As demonstrated in binding and chemotaxis studies, CCR11 is a chemokine receptor that recognizes ligands in the MCP family. The primary ligands for CCR11 are MCP-4 and MCP-2, based on binding affinities and agonist properties in chemotaxis experiments. Other MCP family members also interact with CCR11 with lower affinities. Although CCR11 is most closely related to CCR7, it does not interact with the CCR7 ligands ELC and SLC.

The MCPs share high homology with each other (56–72%) and form their own branch of the CC chemokine family tree. In addition, the MCPs share some functional similarity and are all closely linked on human chromosome 17q11.2 (29). However, MCP expression patterns are distinct, with MCP-4 being expressed constitutively in lung, small intestine, and colon (30, 31), whereas MCP-1 is expressed primarily in cells stimulated with proinflammatory agents (32, 33). MCP-4 has been identified previously as an agonist for CCR2 and CCR3 (30, 31). MCP-2 is recognized by CCR1, CCR2, CCR3, and CCR5 (34–36). MCP-1 is the strongest ligand for the receptor CCR2 (37), and this receptor also recognizes MCP-2 (34), MCP-3 (38), and MCP-4 (30, 31). The characterization of MCP family members as ligands for CCR11 adds additional complexity and redundancy to this diverse repertoire of chemokine functions.

Identification of ligands for orphan GPCRs can be complex. GPCRs can exhibit paradoxical behavior, particularly transfected recombinant receptors. Although not well understood, such unusual behavior may be caused by inappropriate G protein usage, overexpression of recombinant receptors, or other as yet unidentified phenomena. Our laboratory has noted that some chemokine receptors may not be expressed in a stable manner and that functional responses can be lost if not selected for repeatedly.[2] Overexpression is a natural consequence of using a strong promoter and may lead to functional responses that are potentially deleterious to transfected cells. Some changes we have observed with GPCR transfectants are increases in cell adhesiveness or decrease in growth rate.[3] With CCR11 our transfected cell population was initially selected by chemotaxis. When these cells were cloned, the majority had lost their responsiveness to MCP-4, but some clones responded even more vigorously than the original selected population. Thus, chemotactic selection greatly aided our identification and characterization of CCR11.

Compared with other characterized chemokine receptors, we observed only weak calcium mobilization in response to MCP-4 stimulation. Perhaps CCR11 signal transduction is linked to G proteins that are not well complemented in L1.2 cells. Perhaps this receptor does not naturally induce a strong calcium response, like some other GPCRs. Alternatively, CCR11 calcium responses in L1.2 cells may be linked to cellular toxicity. Finally, CCR11 may recognize other, as yet unidentified, ligands that cause more significant calcium flux. Nevertheless, MCP-4

[2] C. J. Raport and P. W. Gray, unpublished observations.
[3] V. L. Schweickart, B. Steiner, and P. W. Gray, unpublished observations.

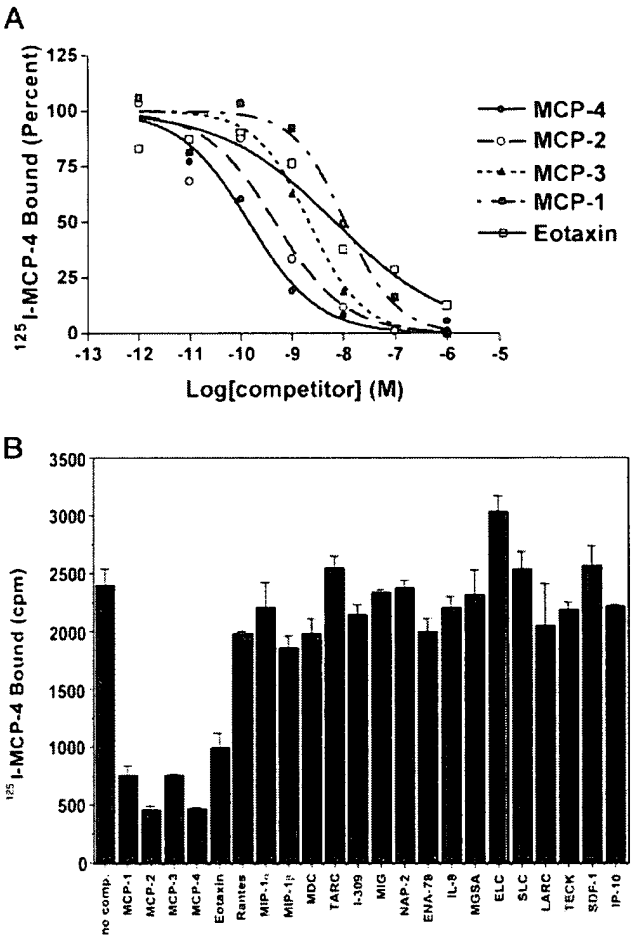

FIG. 6. **Binding characteristics of ^{125}I-MCP-4 to CCR11.** *Panel A*, displacement of the binding of ^{125}I-MCP-4 to CCR11-transfected L1.2 cells with unlabeled MCP-4 (●), MCP-2 (○), MCP-3 (▲), MCP-1 (□), and eotaxin (□). Cells were incubated with 0.1 nM ^{125}I-MCP-4 in the presence of the indicated concentrations of unlabeled chemokine. Cells were washed three times in binding buffer, and the amount of bound ^{125}I-MCP-4 was determined. *Panel B*, displacement of ^{125}I-MCP-4 by other chemokines. L1.2 cells stably transfected with human CCR11 were incubated with 0.1 nM ^{125}I-MCP-4 in the presence of a 100 nM concentration of the indicated chemokines. Cells were washed, and specific binding of ^{125}I-MCP-4 was determined.

is a major ligand for CCR11 as shown by its strong binding affinity and potent agonist activity in chemotaxis experiments.

As shown by Northern blot analysis, CCR11 has an unusual pattern of expression for a chemokine receptor. Because it is not highly expressed in lymphoid organs such as thymus or spleen, CCR11 is not likely to be involved in lymphocyte development as are CCR4, CCR7, and CCR9 (Refs. 10–15). In addition, CCR11 is virtually undetectable in peripheral blood, being primarily expressed in the heart, small intestine, and lung. With the exception of CXCR4, which is broadly expressed in many tissues, chemokine receptors are typically expressed exclusively on cells of lymphoid or myeloid origin. Our inability to detect transcript in these cells may indicate that CCR11 is expressed on a subpopulation of lymphoid cells that are rare in whole blood but resident in specific tissues. CCR3, for example, is expressed only on eosinophils and a subset of Th2 cells and is undetectable by Northern blot in peripheral blood (39, 40). Alternatively, CCR11 may be expressed on parenchymal cells and play a role currently unappreciated for chemokine receptors. Although chemokines and chemokine receptors are generally known for their role in immune cell development and trafficking, there is preliminary evidence that they may have functions outside of the immune system. Knockout experiments have shown that CXCR4 is essential for normal development of the heart, small intestine and brain (16, 41). In addition, Streblow *et al.* (43) have recently proposed that the virally encoded chemokine receptor US28 may play a role in the migration of smooth muscle cells seen in cytomegalovirus exacerbation of vascular disease (43).

Based on its expression pattern, CCR11 may function in cells that are resident in highly vascularized tissues. MCP-1 and CCR2 have previously been associated with atherogenesis and are thought to play a role in recruitment of macrophages to initiate atherosclerotic plaque formation (18, 44). Vascular expression of MCP family members results in CCR2-mediated monocyte recruitment and macrophage development; this may be accompanied by CCR11-mediated events. Perhaps CCR2 and CCR11 complement each other in vascular processes such as remodeling of the vessel wall to accommodate monocyte influx. The complex redundancy of MCP chemokines and their receptors in the vasculature may help to explain the results of transgenic and gene knockout studies (18, 42, 44, 45). These gene alterations are not lethal and often do not have severe complications on their own, suggesting a compensatory role of genes with similar function. Further studies with CCR11 and its ligands will be required to understand fully their roles in health and disease.

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APPENDIX A

gi|7706769|ref|NP_057641.1| orphan seven-transmembrane receptor, chemokine related [Homo sapiens]
gi|14285406|sp|Q9NPB9|CKRB_HUMAN C-C chemokine receptor type 11 (C-C CKR-11) (CC-CKR-11) (CCR-11) (Chemokine receptor-like 1) (CCRL1) (CCX CKR)
gi|7274392|gb|AAF44751.1| CC chemokine receptor [Homo sapiens]
gi|7328552|gb|AAF59827.1|AF110640_1 orphan seven-transmembrane receptor [Homo sapiens]
gi|7363342|gb|AAF61299.1|AF193507_1 chemokine receptor [Homo sapiens]
Length = 350

Score = 649 bits (1673), Expect = 0.0
Identities = 350/350 (100%), Positives = 350/350 (100%)

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            MALEQNQSTDYYYEENEMNGTYDYSQYELICIKEDVREFAKVFLPVFLTIVFVIGLAGNS
Sbjct: 1   MALEQNQSTDYYYEENEMNGTYDYSQYELICIKEDVREFAKVFLPVFLTIVFVIGLAGNS 60

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